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The Crustacean Androgen: A Hormone in an Isopod and Androgenic Activity in Decapods[1]

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SYNOPSIS. **The androgenic gland has been described in a variety of crustacean species—isopods, amphipods and decapods. It has been shown to play a role in the regulation of male differentiation and in the inhibition of female differentiation. Upon its application for endocrine manipulation, it inhibits female characteristics. Recently, the androgenic hormone from the isopod *Armadillidium vulgare* was purified and characterized on the basis of a morphological bioassay. The hormone is a glycosylated protein composed of two peptide chains connected each to the other by two disulfide bridges. The pro-hormone consists of the same two chains connected by a third peptide in a complex that resembles the insulin super family hormones. The study of the androgenic gland in decapods lags behind that in the isopods, and a decapod androgenic hormone has yet to be identified. In this review, five decapod species are described as models, in which the androgenic gland exerts morphological, anatomical, physiological and behavioral effects. These models could serve as the basis of possible bioassays for the study of the structure and mode of action of the androgenic hormone in decapod crustaceans.**

INTRODUCTION

Over half a century ago, Cronin (1947) discovered the androgenic gland in the decapod crustacean, the crab *Callinectes sapidus*. Seven years later, Charniaux-Cotton (1954) suggested the involvement of this gland in the regulation of male differentiation and spermatogenesis in an amphipod, as was later confirmed in the isopod *Armadillidium vulgare* (Legrand, 1955). This latter species has subsequently become the main focus of research on the androgenic gland in crustaceans. Today, the androgenic gland is known in many crustaceans, and a great deal of information has been published on its function in crustacean species (see previous reviews: Charniaux-Cotton and Payen, 1988; Sagi, 1988; Payen, 1990; Sagi *et al.*, 1997). In the past decade, the morphological and physiological effects of the gland have been described in decapod crustaceans (Taketomi *et al.*, 1990, 1996; Sagi *et al.*, 1997; Fowler and Leonard, 1999; Khalaila *et al.*, 1999), but the hormone responsible for these effects has not been identified. In parallel, research on the androgenic gland in *A. vulgare* reached a peak at the end of the millenium, with the purification, characterization, sequencing and cloning of an androgenic gland hormone (AGH) (Okuno *et al.*, 1997, 1999; Martin *et al.*, 1998, 1999). In this paper, we summarize the recent advances in the identification of the AGH in the isopod *A. vulgare* and describe the most common models that could be used in the future as bioassay systems to facilitate the study of the AGH—its functions and mode of action—in decapod crustaceans.

The androgenic hormone in the isopod crustacean A. vulgare

The main discoveries regarding the role of the androgenic gland in sex differentiation in the isopod *A. vulgare* were made in the late 1950s and early 1960s (Legrand, 1955; Katakura, 1961; Juchault and Legrand, 1964). Since then, its masculinization effect in females has been thoroughly researched (Legrand *et al.*, 1968; Katakura and Hasegawa, 1983; Hasegawa *et al.*, 1993): it was found to masculinize both pri-

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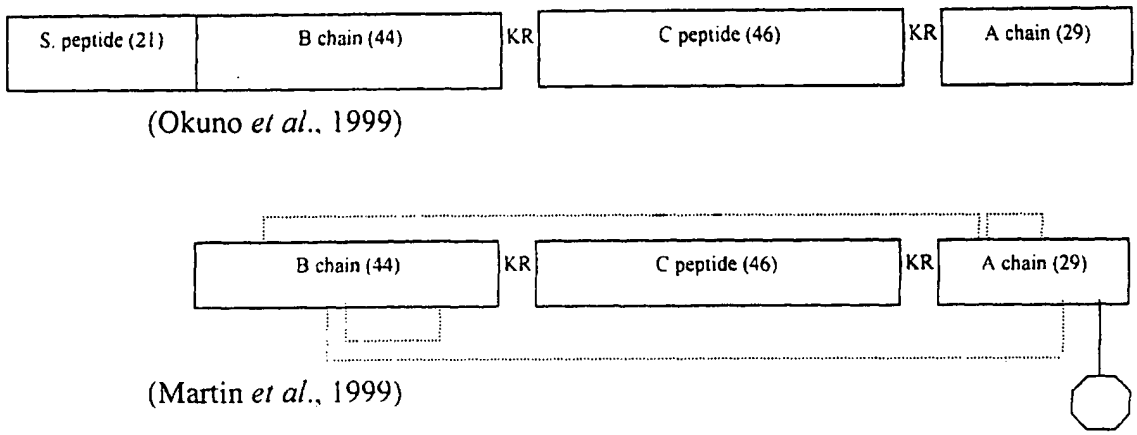

FIG. 1. Schematic structure of the AGH of *Armadillidium vulgare* found independently by two research groups in 1999. The amino acid sequences of subunits A, B and C are similar. (S. peptide) indicates signal peptide. KR = lys-arg. Dashed lines indicate intra- and inter-chain disulfide bridges. O indicates a glycan moiety.

mary and secondary sexual characteristics in *A. vulgare* (Legrand, 1955; Katakura, 1961). Suzuki and Yamasaki (1997) found that the first morphological evidence of masculinization after the implantation of a fresh androgenic gland into a young female *A. vulgare* was the development of male-like first endopodites. Repeated implantation of ethanol-treated androgenic glands into young females at different stages of sexual development caused gonad sex reversal at critical stages (Suzuki and Yamasaki, 1998). Since such glands are not active, this experiment was able to show the exact dose and time at which the hormone exerts its masculinizing effect.

On the basis of the earlier work of Legrand and Katakura (Legrand *et al.*, 1968; Katakura and Hasegawa, 1983), Martin and his team in France (Martin *et al.*, 1999) and Nagasawa, co-workers and their teams in Japan (Okuno *et al.*, 1999) have recently completed the purification, identification and full DNA sequencing of the AGH of *A. vulgare*. For these studies, active material was isolated from hypertrophied androgenic glands of *Wolbachia*-parasitised males (Martin *et al.*, 1990) or from androgenic glands from normal males (Katakura *et al.*, 1975; Juchault *et al.*, 1978; Hasegawa *et al.*, 1987). Polyclonal antibodies were raised against the active material to assess the correlation between the presence of AGH and its biological activity, and to determine AGH concentrations in extracts of whole reproductive systems (Hasegawa *et al.*, 1991). On the basis of the immunoassay of Hasegawa *et al.* (1991), the major HPLC-separated component of the androgenic gland extract, designated AGH I, was further purified by RP-HPLC separation into at least four peaks; three of the peaks were found by ELISA to be immunoreactive to the previously raised antiserum. However, Western blot analysis clearly showed that immunoreactive materials could be detected only in an extract of seminal vesicle and vas deferens and not in an androgenic gland extract (Nagasawa *et al.*, 1995).

Only in the past three years has a breakthrough been achieved in the identification of the hormone. Complete amino acid sequencing of the *A. vulgare* AGH showed a structure that seems to be a pro-AGH in the form of a protein containing three peptide chains, designated A chain, B chain and C peptide (Fig. 1). The complex comprising B chain, C peptide, and A chain, in that order—suggested to be the AGH precursor—is analogous with the proinsulin superfamily peptides (Martin *et al.*, 1998, 1999; Okuno *et al.*, 1999). Molecular sieve HPLC indicated that molecular mass of AGH to be 11–13 kDa (Okuno *et al.*, 1997, 1999), and MALDI-TOF ms of the AGH fraction from hypertrophied androgenic gland showed a broad peak centered at m/z 11,000 (Martin *et al.*, 1999). Exposure of AGH to heat or trypsin or to reductive car-

boxymethylation indicated that the hormone is a heat-stable protein with disulfide bond(s) (Okuno *et al.*, 1997). Chain A was shown, by an Edman degradation and mass spectrometry analysis, to be a peptide of 29 amino acids (Martin *et al.*, 1999; Okuno *et al.*, 1999). There is evidence that N-glycosylation occurred on residue 18 of chain A, which is an asparagine. Chain B gave a clear sequence of 44 amino acids. Chain A and chain B each contain a single intra-chain disulfide bridge and the two chains are linked by two inter-chain disulfide bridges (Fig. 1). The C peptide that links chains A and B in the pro-hormone is made up of a 45 amino acid sequence. Martin *et al.* (1999) purified three active materials from hypertrophied androgenic glands. Of these, two glycoforms, AH_1 and AH_3, were found to be comprised of identical A and B chains of 29 and 44 amino acids, respectively, but different glycan moieties. AH_3, was composed of chain A, chain B and the 45-amino-acid chain C-peptide. The 16.5-kDa AH_3 was therefore suggested to be the precursor of the androgenic hormone. This material was found to be glycosylated and showed AGH androgenic activity (Martin *et al.*, 1999; Okuno *et al.*, 2001). The molecular mass of mature AGH without the carbohydrate moiety was calculated to be 8.7 kDa (Okuno *et al.*, 1999). Based partly on the amino acid sequencing work of Martin *et al.* (1999), Okuno *et al.* (1999) designed two sets of degenerated primers for nested PCR, which resulted in a 317 bp cDNA fragment using the total RNA of the androgenic gland as a template. After the sequencing of the 317 bp fragments, new AGH specific primers were designed, whose PCR amplification was a 501 bp cDNA. The cDNA included open reading frame encoding 144-amino-acid residue proteins. The first 21 amino acid residues were found to be typical of the secretory signal peptide (Fig. 1), indicating that AGH is a secretory peptide (Okuno *et al.*, 1999). The complete structure of the AGH reported by the group of Nagasawa (Okuno *et al.*, 1999) confirmed the structure presented by Martin *et al.* (1999) of chains A, B, and C, the intra- and inter-chain disulfide bridges, and a glycan moiety at Asn18. A molecular probe in a Northern bolt analysis gave the 0.8-kb hybridization signal found exclusively in the RNA preparation from *A. vulgare* androgenic gland (Okuno *et al.*, 1999).

Bioassays for androgenic activity in decapod crustaceans

The first active molecules suggested to be associated with the androgenic gland in decapod crustaceans were terpenoids and steroids. The terpenoids, farnesylacetone and hexahydrofarnesylacetone, were found in the crab *Carcinus meanas* (Ferezou *et al.*, 1978). A steroidogenic nature for the gland was suggested in the freshwater prawn *Macrobrachium rosenbergii* (Veith and Malecha, 1983). Based on the elucidation of the isopod AGH and in keeping with some histological findings in decapods (King, 1964; for review see Sagi, 1988), it is currently common belief that the androgenic hormone in decapod crustaceans is a protein—yet to be purified, and that it is similar to the *Armadillidium* AGH (Sun *et al.*, 2000; Zhang *et al.*, 2000). Attempts to cut short the classical purification process of the androgenic hormone in decapods by means of molecular techniques leaning on the recently published *Armadillidium* sequence are currently under way in several laboratories and could prove fruitful. However, specificity tests in isopods suggest that the hormone is species specific (Martin and Juchault, 1999). A conclusive result will still depend on a reliable bioassay demonstrating clear androgenic activity in analogy with the success story of the identification, purification and cloning of the AGH in isopods, which was based on the reliable bioassay developed by the groups of Legrand and Katakura (Legrand *et al.*, 1968; Katakura and Hasegawa, 1983). Indeed, the fact that research on the hormone in decapods lags behind that in isopods is due in part to the lack of such a bioassay.

Here, we review five possible decapod models that may be used to study the androgenic hormone. In each model organism, the androgenic gland has been identified, and its effects on primary and secondary sexual characteristics have been described (Table 1). Touir (1977) described the effects of the androgenic gland on both

TABLE 1. *Effects of the androgenic gland on primary and secondary sexual characteristics in five model organisms serving as possible bioassays for androgenic activity in decapod crustaceans.*

Species	Primary sexual characteristics	Secondary sexual characteristics	Source
Leander serratus		Development of pleopods	Touir, 1977
Macrobrachium rosenbergii	Gonadal sex reversal	Development of *appendix masculina*, claw morphology, morphotypic differentiation	Nagamine *et al.*, 1980a,b; Sagi *et al.*, 1990
Procambarus clarkii	Inhibition of vitellogenesis	Development of a first abdominal appendage	Nagamine and Knight, 1987; Taketomi and Nishikawa, 1996
Cherax quadricarinatus	Inhibition of vitellogenesis; maintenance of spermatogenesis	Development of red patch on the propodus, inhibition of development of oosetae, aggressive behavior	Khalaila *et al.*, 1999; Karplus *et al.*, 2000
Cherax destructor	Inhibition of vitellogenesis	Inhibition development of the oosetae	Fowler and Leonard, 1999

primary and secondary male characteristics in a number of gonochoristic and hermaphroditic decapod crustaceans. A clear effect on an external characteristic that could serve as a bioassay was described in the shrimp *Leander serratus*: removal of the androgenic gland, accompanied by amputation of the first pleopods, resulted in the regeneration of undifferentiated pleopods. These results suggest that the AGH is essential for the development and maintenance of such secondary male characteristics (Touir, 1977).

In the prawn *M. rosenbergii*, complete sex reversal was achieved by andrectomy of males at an early immature stage, resulting in female differentiation, including the development of ovaries, oviducts and female gonopores (Nagamine *et al.*, 1980*a*; Sagi *et al.*, 1990). Similarly, androgenic gland implantation into early stage immature females lead to the development of testes, sperm ducts and male gonopores (Nagamine *et al.*, 1980*b*). *M. rosenbergii* animals that had undergone sex reversal proved to be capable of mating with normal specimens and producing progeny (Sagi and Cohen, 1990; Malecha *et al.*, 1992). External evidence of masculinization, such as the development of *appendices masculinae*, male copulatory organs, were reported after androgenic gland implantation into females (Nagamine *et al.*, 1980*b*). The process of transformation through the different male morphotypes that coexist in *M. rosenbergii*, such as the orange-claw and the blue-claw morphotypes, was prevented in males andrectomized at critical stages, the effects being evident in both body length and claw morphology (Sagi *et al.*, 1990).

A histological study in the crayfish *Procambarus clarkii* at an early developmental stage showed a high correlation between the differentiation of the androgenic gland and the testes (Taketomi *et al.*, 1996). In a study of the effect of the androgenic gland on the development of secondary sexual characteristics in *P. clarkii*, it was shown that androgenic gland implantation into immature females resulted in masculinization of the first abdominal appendages (Taketomi and Nishikawa, 1996). This procedure also lead to the inhibition of vitellogenesis (Taketomi and Nishikawa, 1996). On the other hand, a study on the organogenesis and differentiation of the genital organs and the androgenic gland in the prawn *Penaeus japonicus* put in question the role of the androgenic gland in the early differentiation of the gonads (Nakamura, 1992; Nakamura *et al.*, 1992).

Experiments on androgenic gland manipulation in the crayfish *Cherax quadricarinatus* confirmed the role of the gland in regulating the development of male secondary sexual characteristics and in the function of the gonads. Intersex specimens of *C. quadricarinatus*, in which one half has an androgenic gland, develop a male reproductive system on the same side. The contra-

lateral half, which lacks the androgenic gland, has a female reproductive system in a permanently arrested state. The secondary external characteristics of such intersex animals are male-like on both sides (Sagi *et al.*, 1996). Androgenic gland ablation in these intersex animals resulted in degeneration of the male reproductive system and in the onset of secondary vitellogenesis in the previously arrested ovarian lobe (Khalaila *et al.*, 1999). In such individuals, secondary sexual characteristics, such as the red patch on the propodus, were degenerated and the pleopods were differentiated to female-like pleopods, complete with feminized ovigerous setae. A quantitative ELISA developed to monitor the onset of secondary vitellogenesis in *C. quadricarinatus* showed a high level of a vitellogenic-specific polypeptide in the hemolymph of androgenic-gland-ablated intersex individuals *vs.* control intact intersex individuals. A low level of the vitellogenic-specific polypeptide was found in the hemolymph of androgenic-gland-implanted females *vs.* control females (Sagi *et al.*, 1999). Androgenic gland implantation into females affected both primary and secondary sex characters (Khalaila *et al.*, 2001), also agonistic behavior of *C. quadricarinatus* implanted females was evaluated by comparing the interactions between males and females. Contests that involved male animals alone were much more intense than those of a male with a female. The aggressive behavior of implanted females was intermediate between that of male/male and male/female with regard to aggressive behavioral features such as the duration of an escalated fight and the duration of grasp (Karplus *et al.*, 2000). In an another species of crayfish, *Cherax destructor,* implantation of the androgenic gland into immature females caused inhibition of the development of oosetae and of secondary vitellogenesis (Fowler and Leonard, 1999).

In the five model organisms described above, the androgenic gland exerts well-defined morphological, behavioral and physiological effects (Table 1). Each of these model organisms, could serve as a basis for the development of a bioassay for the study of the androgenic gland in decapod crustaceans. The bioassay, which should reflect a well-defined effect of the androgenic gland on the gonad or on sexual characteristics, should be short and reproducible. The assay could be either *in vitro* bioassay or a short *in vivo* bioassay, comparable to the bioassay in *A. vulgare*, which led to the isolation of AGH in this species. Thus, the effects of androgenic gland implant should be shown immediately after the post-implantation molt (Katakura and Hasegawa, 1983), or an immediate measurable change should be evoked *in vitro* by the hormone in a target tissue.

The next steps

Unlike the AGH of the isopod *A. vulgare*, the exact nature of the AGH in decapod Crustacea has not yet been identified. Moreover, the morphological, physiological and behavioral effects of the gland observed to date have raised the question whether a "one factor show" for all the above-described effects the applicable in higher crustaceans. The answer to that question is not yet clear. As indicated previously, the AGH of the isopod *A. vulgare* has been purified, and cDNA cloning and expression have been performed. However, the bioassay for the purified substance was the induction of the development of the first endopodite in a female injected with that substance. The effects of the purified AGH on internal sexual characteristics have not yet been determined.

The only androgenic substances purified thus far from the androgenic gland of decapod crustaceans are lipidic in nature. A lipoidal substance with a molecular mass of 200–250 daltons was extracted from the androgenic gland of the crab *Carcinus maenas* (Berreur-Bonnenfant *et al.*, 1973). When injected into an *Orchestia gamarella* sexually active female, that lipoidal substance inhibited vitellogenesis. It also induced the appearance of a carotenoid pigment in the antennae—a secondary male sexual character—of *Talitrus saltatus* females. The active molecule, characterized by Ferezou *et al.* (1978) as farnesylacetone, was shown to be synthesized by the androgenic gland. The action of farnesylacetone in low concentrations is rapid and organ specific (be-

ing expressed in the gonad) but is not species specific. Farnesylacetone affects protein and RNA synthesis in its target organs (Berreur-Bonnenfant and Lawrence, 1984). A histochemical study of Veith and Malecha (1983) demonstrated—in keeping with previous work—that the androgenic gland of *M. rosenbergii* stained positive for lipids. In contrast, a recent histological and histochemical study of Awari and Kiran (1999) described three types of cell in the androgenic gland of *M. rosenbergii* but was not able to demonstrate the presence of lipids in any of the cell types. However, in light of the wide range of effects described for the androgenic gland, the possibility of a non-protein androgenic hormone in crustaceans should not be disregarded.

Recent histological evidence in *M. rosenbergii* supported the idea of a proteinaceous androgenic hormone (Awari and Kiran, 1999). Earlier, the proteinaceous nature of the secretion of the androgenic gland in the crab *Pachygrapsus crassipes* was indicated by the considerable amount of protein in the cytoplasmatic secretory vesicles (King, 1964). Similarly, the ultrastructure of the androgenic gland of *P. clarkii* supports the possibility of a peptidergic-proteinaceous secretion (Miyawaki and Taketomi, 1978; Taketomi, 1986).

The use of the above-described models might open the path to the purification of AGHs in decapod crustaceans. The purified AGH should be further investigated for its responsibility for physiological effects described above. This will enable further research into the mode of action of the androgenic hormone, an issue that has not been addressed in any crustacean species thus far.

In addition to its basic scientific significance, a study of the androgenic gland might present tremendous applied merit in crustacean species exhibiting a sexual dimorphic growth pattern, possibly related to effects exerted by the androgenic gland, as found in the freshwater prawn *M. rosenbergii* (Sagi *et al.*, 1997).

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